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Applicant: Simantov, et al.

Serial No.: 09/730,379

Filed: December 5, 2000

For: THROMBOSPONDIN-BINDING REGION OF HISTIDINE-RICH GLYCOPROTEIN AND METHODS OF USE

Examiner: Misook Yu

Group Art Unit: 1642

Confirmation No: 7403

Docket: 955-7P CON

Dated: January 20, 2004

I hereby certify this correspondence is being sent by First Class Mail, postage prepaid and addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on January 20, 2004

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Commissioner for Patents
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Alexandria, VA 22313-1450

DECLARATION OF ROY L. SILVERSTEIN UNDER 37 C.F.R. §1.132

Sir:

I, Roy L. Silverstein, M.D., a co-inventor of the above-identified U.S. patent application, declares as follows:

1. I am Chief of the Division of Hematology, Medical Oncology and Stem Cell Transplantation at the Weill Medical College of Cornell University and New York-Presbyterian Hospital - Cornell Campus.

2. Experiments were conducted under my direct supervision and control for the purpose of demonstrating increased cutaneous angiogenesis and accelerated wound closure in transgenic mice expressing histidine-rich glycoprotein (HRGP). These experiments are described in Exhibit 1. Transgenic mice over-expressing HRGP were generated by transfecting normal mice with additional full length

murine HRGP genes driven by the keratin 14 (K14) promoter in basal keratinocytes (see exhibit 1, paragraph [0001] and figure 1). Two transgenic lines were established (Tg 1 and Tg 19). The Tg 1 line contained seven copies of the transgene and the Tg 19 line contained twelve copies. The skin extracts of the transgenic mice contained a greater than ten-fold increase in HRGP than control (wild-type) mice (see exhibit 1, paragraph [0002] and figure 2). Analysis of the blood vessel of whole mounts of the ears demonstrated an increase in vascular tortuosity and branching in the transgenic mice compared to control mice (see exhibit 1, paragraph [0003] and figure 3). Further, immunohistochemical staining of skin sections from wild-type and transgenic mice with an antibody against the endothelial cell marker PECAM (CD31), showed a greater number of CD31 positive cells in the transgenic mice than in the wild-type mice.

3. The observed increase in vascular tortuosity and branching, and the greater number of CD31 positive cells in the transgenic mice than in the wild-type control mice indicates that the increased expression of HRGP in the skin of transgenic mice leads to increased blood vessel formation (e.g., angiogenesis).

4. The two transgenic mouse lines (Tg 1 and Tg 19) were also utilized to examine the effect of increased expression of HRGP in a wound model *in vivo*. In one wound-healing model, the mice were subjected to full thickness punch wounds. The time to wound closure was measured. One of the transgenic mouse lines (Tg 1) showed accelerated wound closure compared to control mice without the HRGP transgene (see exhibit 1, paragraph [0005] and figure 4). The other transgenic mouse line (Tg 19) did not. The Tg 1 transgenic mouse line expressed more HRGP than both the Tg 19 transgenic mouse line and the control mice.

5. Another model of wound healing is also described in exhibit 1. The mice were implanted subcutaneously with polyvinyl alcohol sponges (see exhibit 1, paragraph [0006]). Analysis of the sponges demonstrated a greater amount of vascularization in the wound granulation tissue of the Tg 1 transgenic mouse line

than in the control mice (see exhibit 1, paragraph [0007] and figures 5 and 6). In addition, a greater number of fine fibrovascular networks was detected in the transgenic mice compared to the control mice (see exhibit 1, paragraph [0007] and figure 7).

6. Results similar to those for Tg 1 transgenic mouse line described in paragraph 5 above were also observed in the Tg 19 transgenic mouse line. Specifically, although to a lesser degree than that of the Tg 1 transgenic mouse line, analysis of the sponges also showed a greater amount of vascularization in the wound granulation tissue of the Tg 19 transgenic mouse line than in the control mice (see exhibit 1, paragraph [0008]).

7. The above results demonstrate that over-expression of HRGP promotes angiogenesis and promotes wound healing.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 1/21/04 Signature: _____
Roy L. Silverstein, M.D.

EXHIBIT 1

INCREASED CUTANEOUS ANGIOGENESIS AND ACCELERATED WOUND CLOSURE IN TRANSGENIC MICE EXPRESSING HISTIDINE-RICH GLYCOPROTEIN (HRGP)

[0001] Transgenic (Tg) mice were generated using the keratin 14 (K14) promoter to drive expression of full length murine HRGP in basal keratinocytes (See Figure 1). Two transgenic lines were established (Tg 1 and Tg 19). The Tg 1 line contained 7 copies of the transgene, as assessed by densitometric analysis of Southern blots. The Tg 19 line contained 12 copies of the transgene. The transgenic mice were viable, breed normally and their skin was normal upon gross examination.

[0002] Western blot analysis of purified skin extracts using a polyclonal anti-HRGP antibody demonstrated a greater than 10-fold increase in HRGP protein in the Tg lines (Figure 2; left panel) compared to wild-type mice (control mice). There was no increase in circulating HRGP in Tg mice as measured by enzyme linked immunosorbent assay of plasma (Figure 2; right panel). Further, there was no increase in HRGP deposition in liver, spleen, muscle, kidney, or brain.

[0003] To determine the effect of HRGP over-expression on normal blood vessel formation, mice were perfused with fluoroscein-conjugated dextran. Blood vessels of whole mounts from the ears were analyzed. As seen in Figure 3A, there was an increase in vascular tortuosity and branching in Tg mice compared to controls. The tortuosity and branching of blood vessels was quantified by measuring numbers of branches per mm of vessel (4.0 ±0.39 in the Tg vs 2.2 ±0.20 branches/mm in controls, $p=0.002$) or by overall branch density (3.33±0.55 branches per vessel in Tg vs. 2.0 ± .08 in control, $p< 0.001$), or by distance between branches (Figurer 3B). With all three methods we saw significantly more branching in the transgenic mice.

[0004] To quantitate vascularity further, immunohistochemical staining of skin sections from wild-type (WT) and Tg mice was performed with an antibody against the endothelial cell marker PECAM (CD31). A greater number of CD31 positive cells was observed in Tg (19.80 ± 2.20 cells/hpf) than in WT (13.05 ± 1.55, $p=0.01$) mice (Figure 3C). These results indicate that

under basal, non-pathological conditions, localized increased expression of HRGP in skin led to increased blood vessel formation.

[0005] To explore the angiogenic response under pathological conditions in this model, we used full thickness punch wounds and measured time to wound closure. The Tg 1 line that expressed the most HRGP closed wounds faster than controls (wild-type mice) (Figure 4).

[0006] Next, another model of wound healing was utilized. Polyvinyl alcohol sponges which were 6mm in diameter and 2mm thick were implanted subcutaneously in the dorsum of the transgenic and wild-type mice. The sponges were then excised 7 days later and analyzed histologically.

[0007] As seen in Figure 5, within the sponges from the Tg 1 mouse line is a vascularized cellular infiltrate that resembles wound granulation tissue. The amount of vascularization in the granulation tissue can be quantified by measuring hemoglobin by Drabkin's method (1.38±0.63 mg/ml in the Tg 1 vs. 0.70±0.15 mg/ml in the controls) (Figure 6). Therefore, the Tg 1 mouse line demonstrated a greater amount of vascularization in the wound granulation tissue that control mice without the transgene. In addition, a greater number of fine fibrovascular networks by histologic examination of hemoglobinized channels was detected (Figure 7) in the Tg 1 mouse line than control mice.

[0008] Similar results were also observed in the Tg 19 transgenic mouse line. Specifically, although to a lesser degree than that of the Tg 1 transgenic mouse line, analysis of the sponges also showed a greater amount of vascularization in the wound granulation tissue of the Tg 19 transgenic mouse line than in the control mice. (Data not shown.)

[0009] These results demonstrate that over-expression of HRGP in the skin leads to increased basal cutaneous vascularity, faster time to wound closure, and increased vascularization of cutaneous wounds. The data suggest that HRGP modulates the balance towards a pro-angiogenic phenotype and are consistent with a role for the CD36/TSP system in regulating cutaneous angiogenesis.

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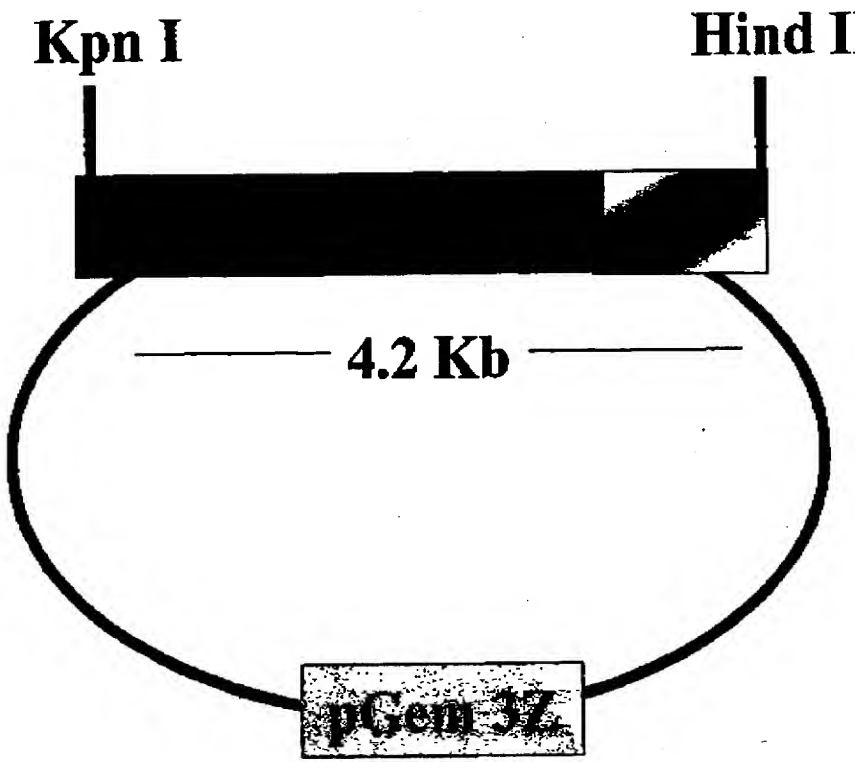
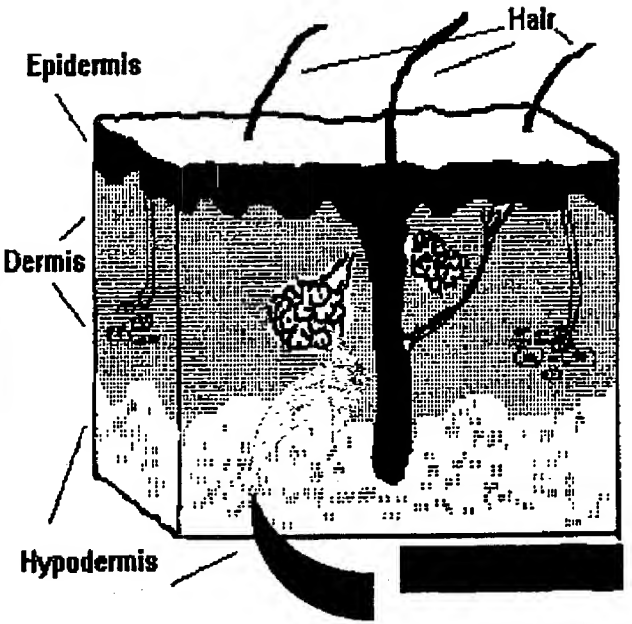
Targeted Overexpression of HRGP in Skin Using a Keratin 14-HRGP Construct





K14 is expressed in basal keratinocytes

FIGURE 1

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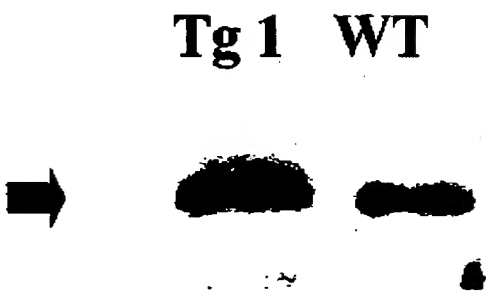
K14-HRGP Tg Skin Extracts Contain Increased Amounts of HRGP Protein But No Increase in Circulating HRGP

Cutaneous HRGP



> 10-fold increase in HRGP protein in Tg lines.

Plasma HRGP

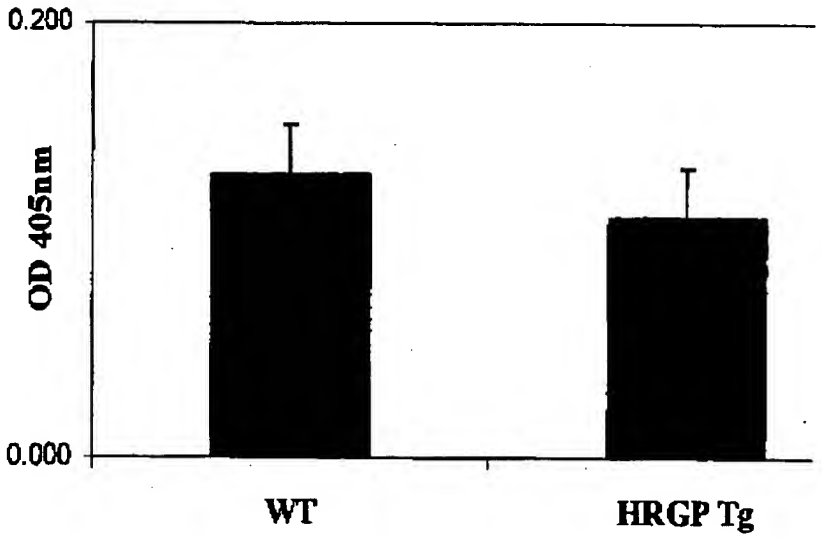


FIGURE 2

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Increased Cutaneous Vascular Tortuousity in Mice with Overexpression of K14-HRGP

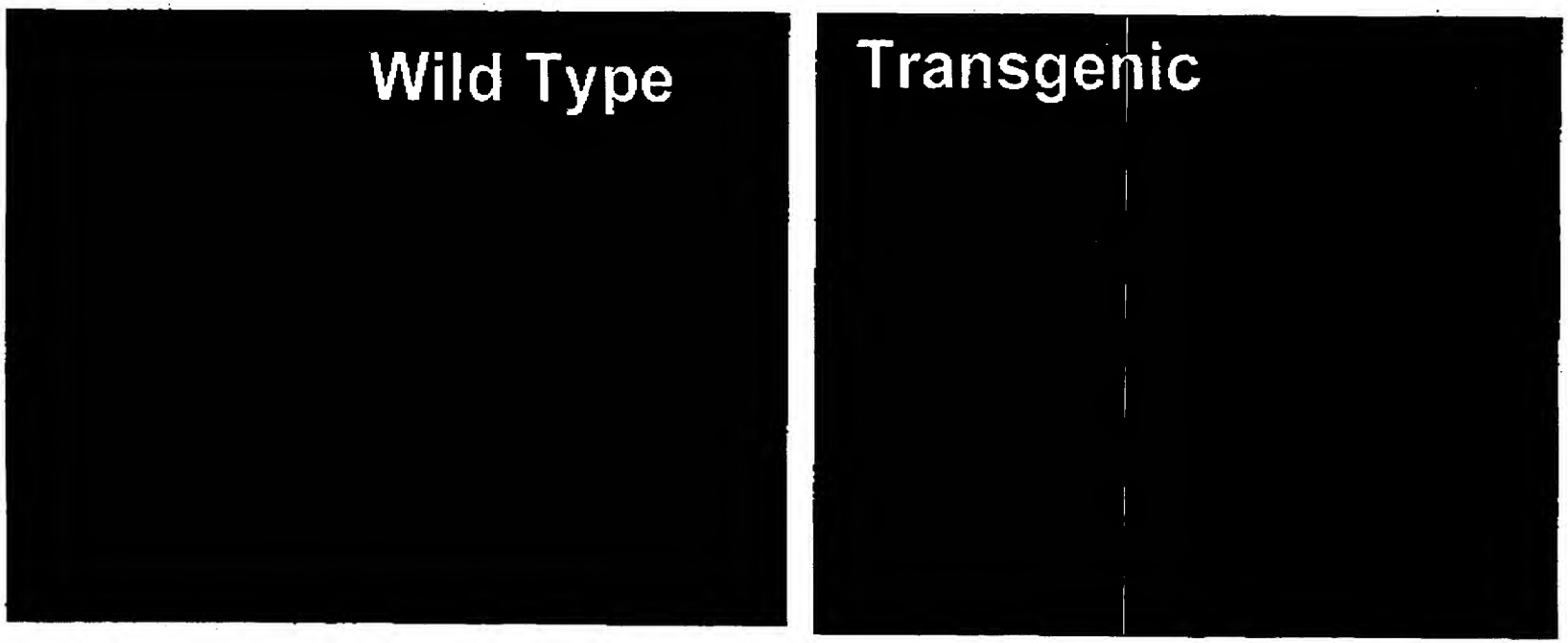

FIGURE 3A

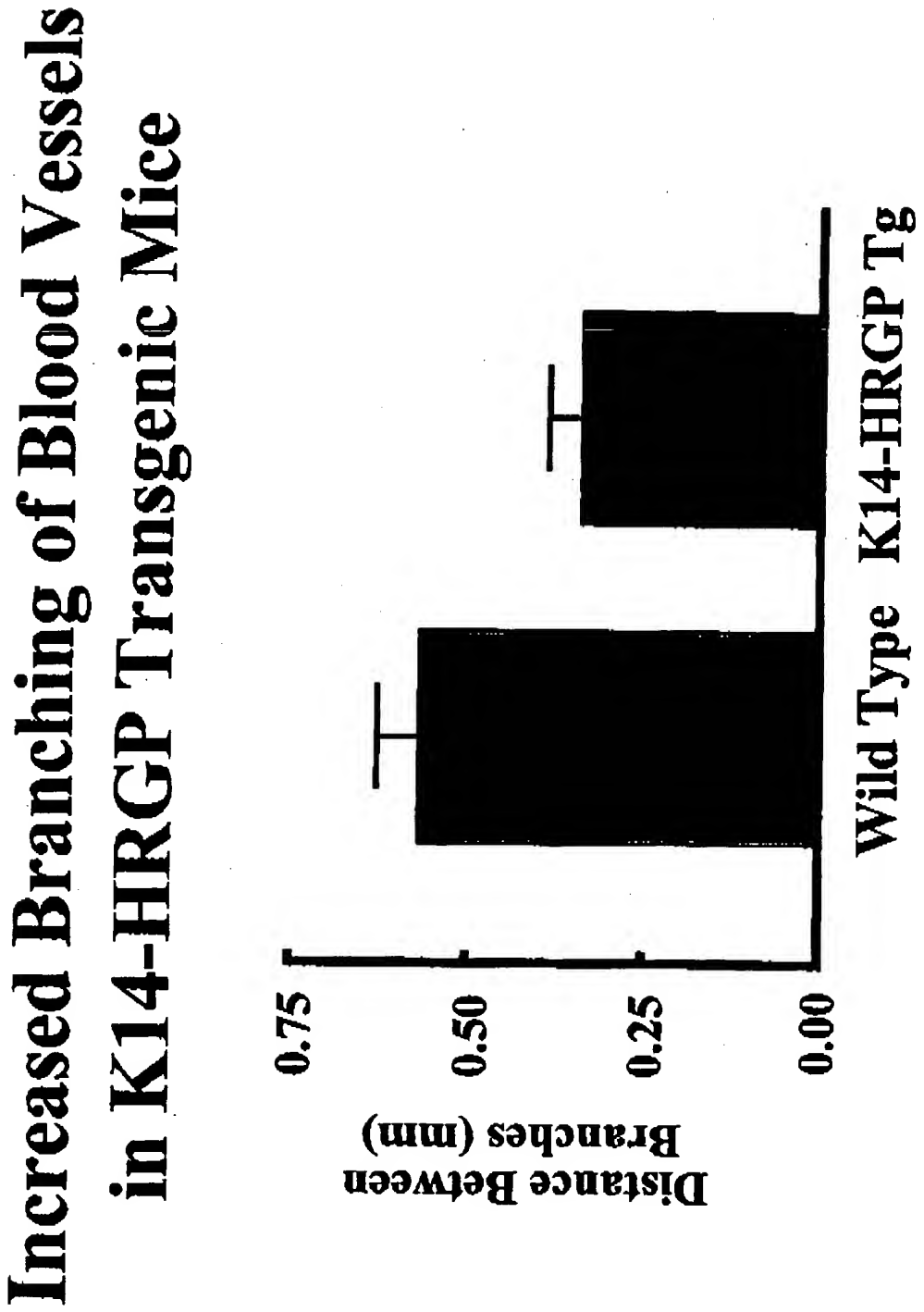
Increased Branching of Blood Vessels
in K14-HRGP Transgenic Mice
Distance Between
Branches (mm)
0.75
0.50
0.25
0.00
Wild Type
K14-HRGP Tg

FIGURE 3B

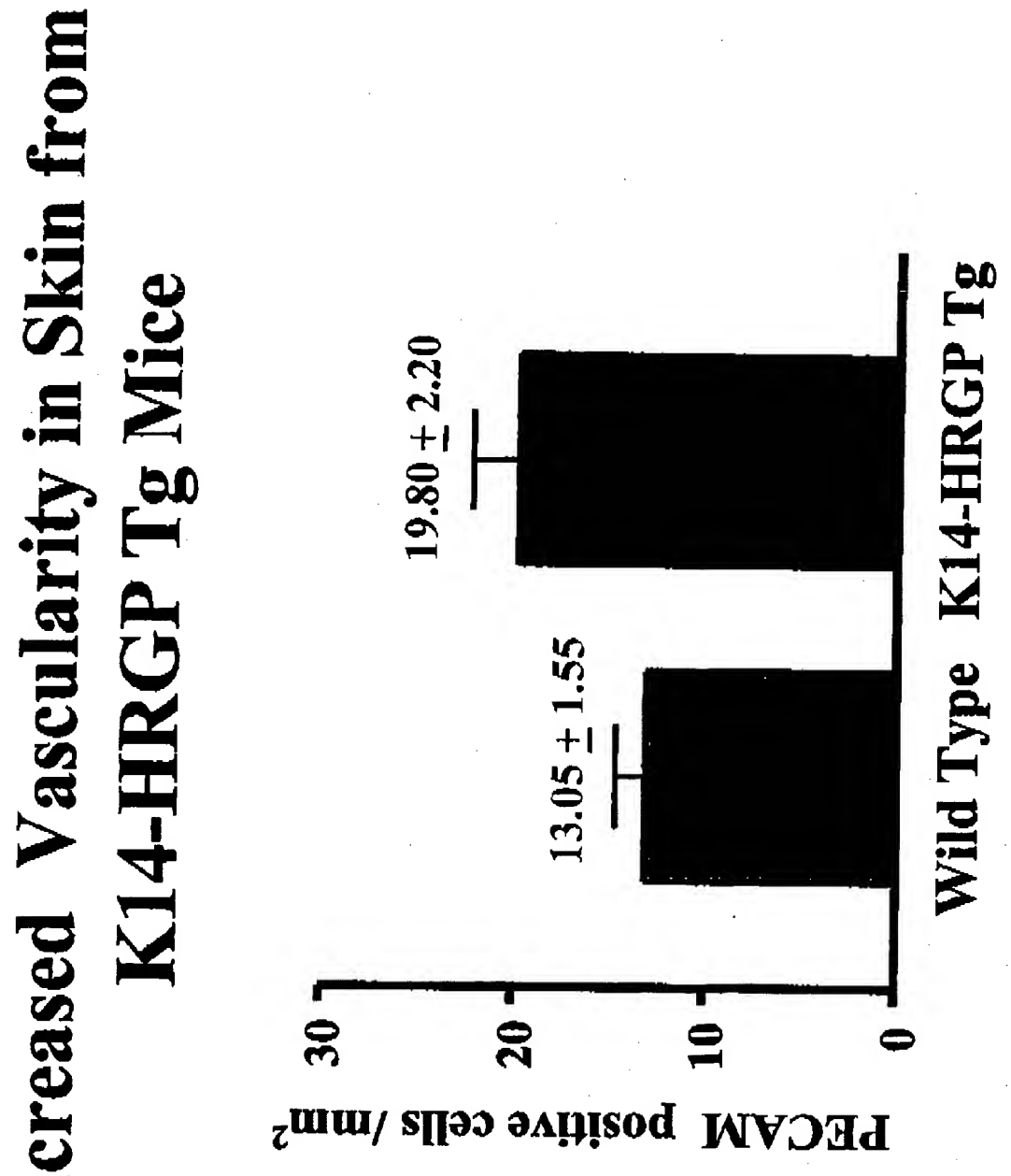
Increased Vascularity in Skin from K14-HRGP Tg Mice
PECAM positive cells /mm2
30
20
10
0
19.80 ± 2.20
13.05 ± 1.55
Wild Type
K14-HRGP Tg

FIGURE 3C

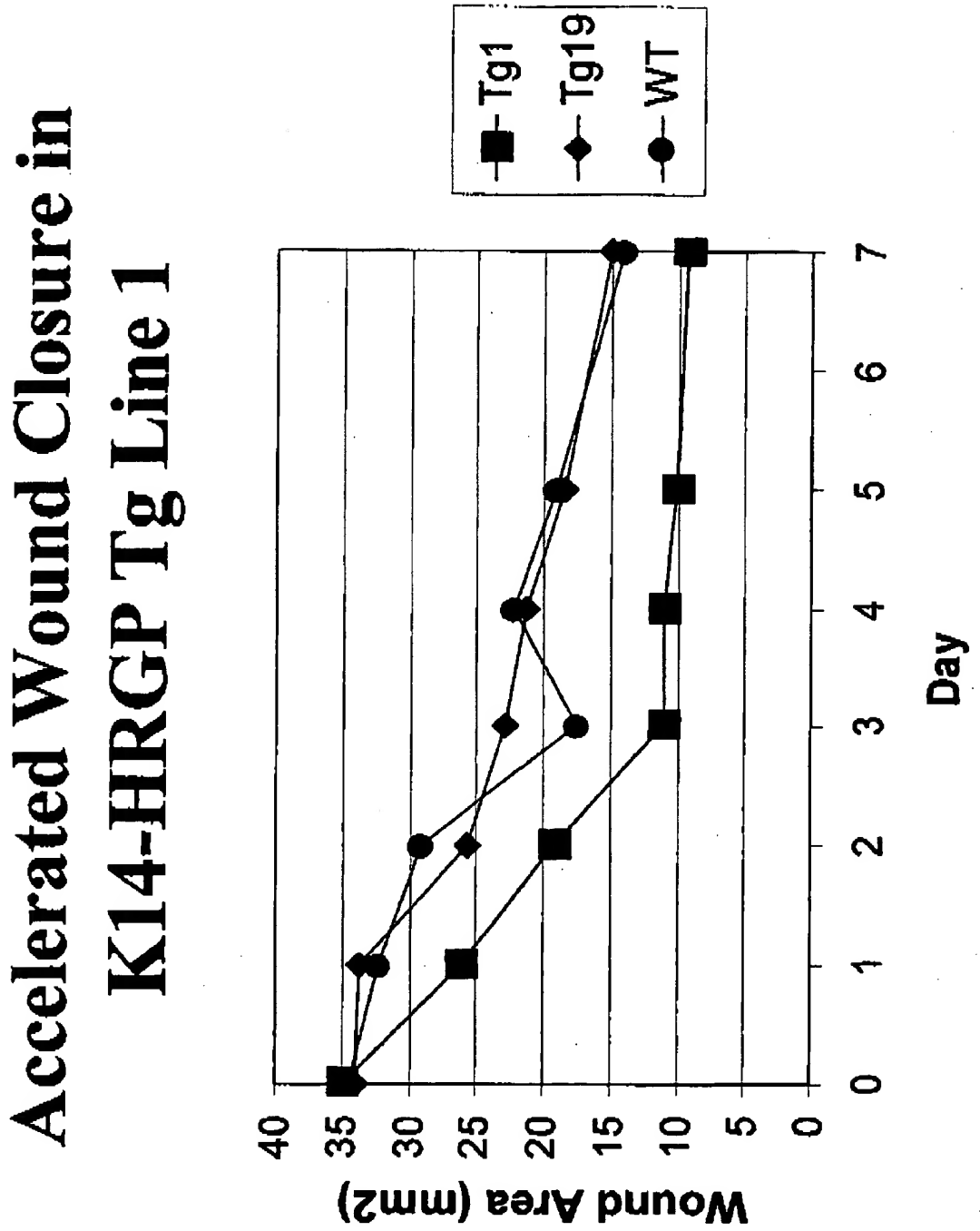
Accelerated Wound Closure in
K14-HRGP Tg Line 1
Tg1
Tg19
WT
Wound Area (mm2)
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Day

FIGURE 4

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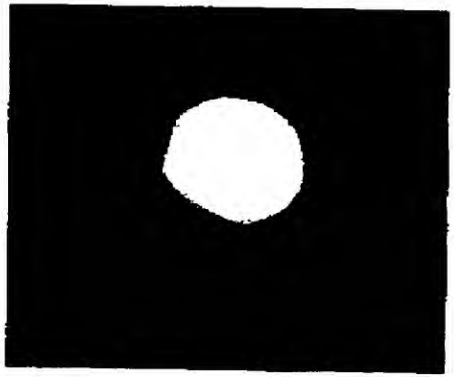
Sponge Model of Wound Healing

6 mm-diameter, 2 mm thick polyvinyl alcohol sponges were implanted subcutaneously



H and E staining of sponge excised after 7 days reveals cellular infiltration mimicking a wound healing response

FIGURE 5

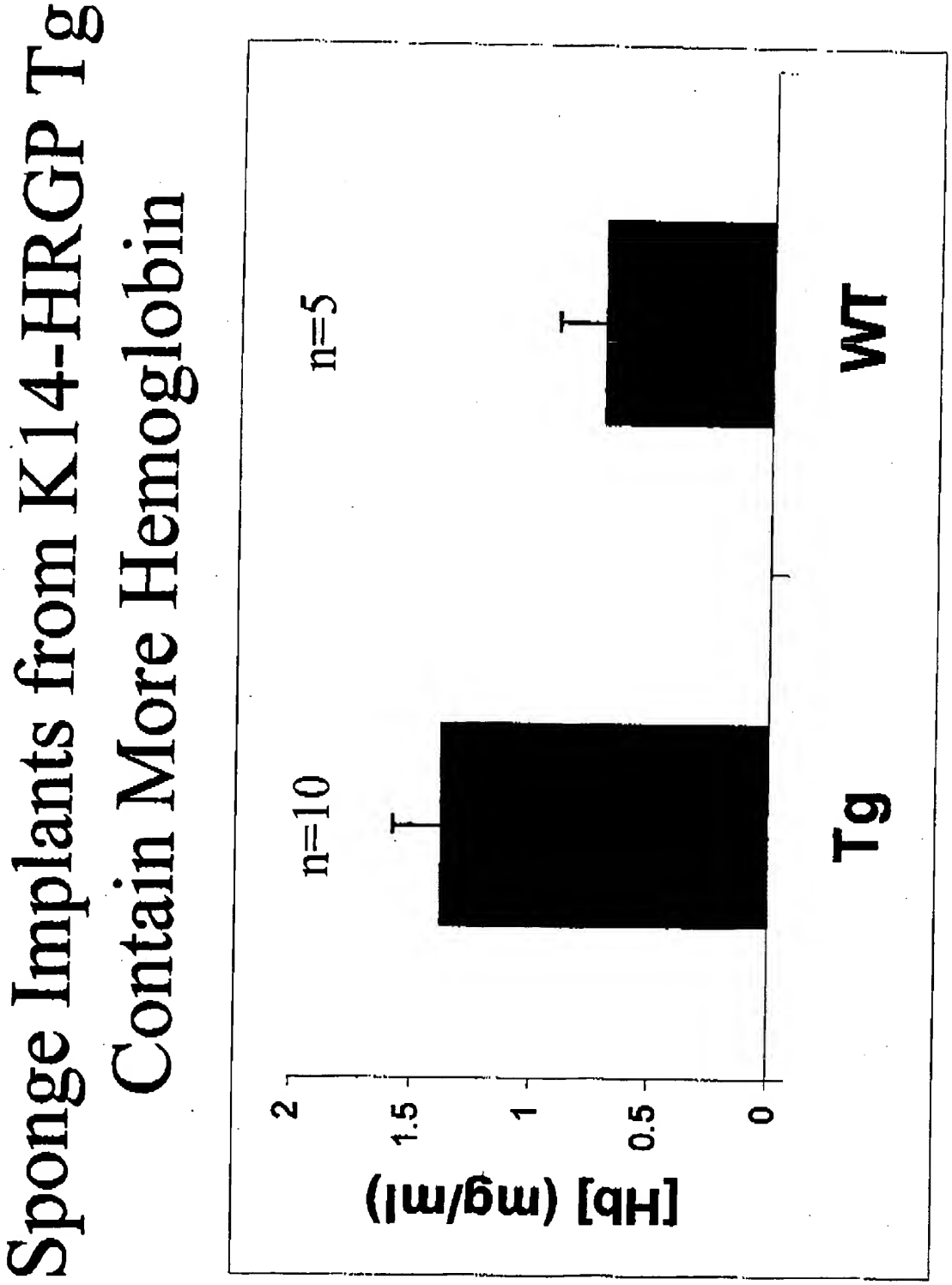

Sponge Implants from K14-HRGP Tg Contain More Hemoglobin
[Hb] (mg/ml)
2
1.5
1
0.5
0
n=10
n=5
Tg
WT

FIGURE 6

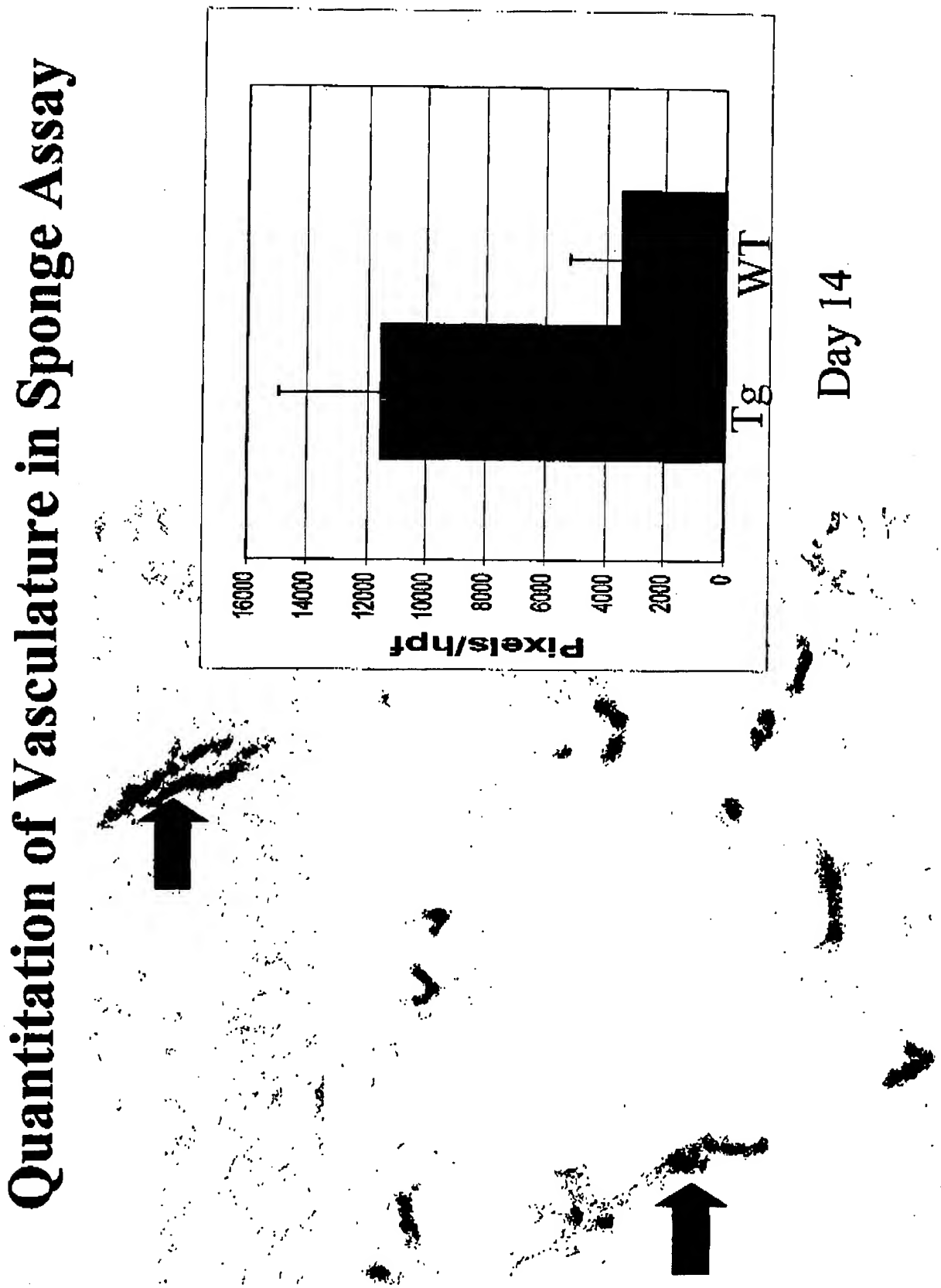

Quantitation of Vasculature in Sponge Assay
Pixels/hpf
16000
14000
12000
10000
8000
6000
4000
2000
0
Tg
WT
Day 14

FIGURE 7